CORRELATIONS IN THE COLON-AEROGENES GROUP *

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Recent papers by Rogers and Clark and their associates, and those of Levine and others, have done much to create a new interest in the classification of organisms in the colon-aerogenes group. The correlation of specific characters of members of this group with their habitat has been sought for some time on account of its sanitary significance, but until recently no progress has been made which has attracted any considerable degree of attention.

Harden[1] and MacConkey[2] have discussed the action of B. cloacae and B. [lactis] aerogenes on the sugars, particularly glucose, and noted the infrequency with which these 2 organisms were met in fecal matter. Both of these investigators observed the extensive utilization of glucose by these organisms as compared with the colon bacillus proper, and stated that in most instances the glucose was completely exhausted from the culture medium by B. cloacae and B. [lactis] aerogenes, in distinction from the other members of the entire gas-forming group. They also pointed out the correlation of this property with the Voges and Proskauer reaction, which none of the other organisms under observation exhibited. In fact the Voges and Proskauer reaction was thought at this time to be an easy means of distinguishing between the members of the group which are of fecal and those which are of non-fecal origin.

Rivas[3] in discussing the so-called 'saccharolytic group' of sugar-fermenting bacteria, which includes B. cloacae and B. [lactis] aerogenes, states that since the colon bacillus is not a member of this group it should not be employed for the removal of muscle sugar from meat extract, in the preparation of nutrient broth, but that 1 of the organisms which cause rapid and complete decomposition of glucose and glucose-like sugars, namely the saccharolytic group, should be used. Kendall, Day and Walker[4] have likewise shown that the colon bacillus differs in certain important respects from B. cloacae, aside from gelatin liquefaction. B. cloacae ferments glucose broth, giving a CO_2:H ratio of 2:1, as was first shown by Smith.[5] Furthermore, members of the cloacae group decompose glucose rapidly, the carbohydrate disappearing from the culture medium within 24 hours. The colon bacillus, under similar con-

* Received for publication February 29, 1917.

[1] Jour. Hyg., 1905, 5; p. 488. Proc. Roy. Soc., 1906, B. 77, p. 424. Ibid., 1906, B. 77, p. 399.

[2] Jour. Hyg., 1906, 6, p. 385. Ibid., 1909, 9, p. 86.

[3] Jour. Med. Research, 1908, 18, p. 81.

[4] Jour. Am. Chem. Soc., 1913, 35, p. 1227.

[5] Wilder Quarter Century Book, 1893, p. 212.

ditions, produces a much larger amount of acid than B. cloacae, the acid of the former being of sufficient strength to cause complete cessation of growth.

These suggestions received little attention until Rogers, Clark, and Davis[6] by the methods of exact gas analysis made a critical study of the colon-aerogenes group and found that in general 2 subgroups of organisms existed, namely the 'high ratio' or cloacae division, and the 'low ratio' or colon subgroup. These could be subdivided further into 6 lower divisions, as was pointed out by Rogers, Clark, and Evans.[7] These same investigators[8] when studying the bacteria of bovine feces, observed that only 1 of 150 strains of gas-formers gave a gas ratio which identified it with the high ratio organisms.

Clark and Lubs[9] showed that the bacteria of the high and low ratio groups could be distinguished from each other readily by the hydrogen-ion concentration[10] resulting from the fermentation of glucose in a medium of the following composition:

Witte's peptone	5 gm.
Glucose	5 gm.
K_2HPO_4	5 gm.
Distilled water	1000 c.c.

Levine[11] in his study of the newly proposed methyl-red test observed that the organisms which gave no red coloration in Clark and Lubs medium were also those which were positive by the Voges and Proskauer test, and that none of the methyl-red positive organisms gave the Voges and Proskauer reaction.

Levine's observations were apparently confirmed by Hulton[12] and Greenfield,[13] who regard these correlations as being of much sanitary significance.

The work of Prescott and his pupils,[14] Papastiriu,[15] Metcalf, Winslow and Walker,[16] Fromme,[17] Rogers, Clark, and Evans,[7] Johnson,[18] and others has indicated that gas-forming bacteria of the colon group type are found in nature in places where there has in all probability been no recent fecal contamination.

In 1914 it was decided by one of us to make an exhaustive study of such organisms, particularly those which are found in the soil, and to endeavor to classify them. The work extended over 2 years, and while it is far from complete in certain respects, the results may prove to be of some assistance to others who are engaged in similar studies.

[6] Jour. Infect. Dis., 1914, 14, p. 411.
[7] Jour. Infect. Dis., 1914, 15, p. 99.
[8] Ibid., 1915, 17, p. 137.
[9] Ibid., p. 160.
[10] The hydrogen-ion concentration was determined after 3-5 days' incubation at 30 C., methyl red being used as the indicator.
[11] Jour. Bact., 1916, I, p. 153. Jour. Infect. Dis., 1916, 18, p. 358.
[12] Ibid., 19, p. 606.
[13] Ibid., p. 647.
[14] Biological Studies by Pupils of W. T. Sedgwick, Boston, 1906, p. 208.
[15] Arch. f. Hyg., 1902, 41, p. 439.
[16] Science, 1905, 26, p. 797.
[17] Ztschr. f. Hyg. u. Infectionskrankh., 1910, 65, p. 251. Ibid., 1913, 74, p. 74.
[18] Abstr., Jour. Bact., 1916, 1, p. 96.

METHODS OF TITRATION

The titration methods of various workers were first investigated. The absence of uniformity in the method of procedure became very apparent, and it is not surprising that results of different investigators should be in some instances so conflicting. Most of the titration experiments have been conducted on both fresh media and culture fluids of different ages.

From the available data it was seen that there is a tendency toward the hot titration and the subtraction of a blank titration, in order to determine the amount of acid formed. Is this the correct method? Before attempting to answer this question let us first consider the significance of the term 'acidity.'

If the hydrogen-ion concentration in a culture is a growth function of the ability of the organism to resist its own acid, and is due to no other cause, we would expect to obtain the same acidity with all sugars which a given organism can ferment with the production of acid. This is seldom observed in practice, however, for instances of high acidity in one sugar and low in another are common. It is recognized that a sufficient amount of acid will ultimately cause the death of an organism, or complete cessation of all its activities, but unless death does ensue it is difficult to believe that the whole organism is affected in such a way that no more acid can be produced. Rather would the cause of the failure to produce any more acid be looked for in the inhibiting effect of the end-product on the particular biologic mechanism which brings its formation about. Present day views on the action of enzymes would favor the idea that an equilibrium would be reached. Such equilibrium may or may not coincide with the acid death point of Holman.[19]

This view is not materially different from that enunciated by Hopkins and Lang,[20] but it is evident from their subsequent work that they disregarded it.

Finally, the principles of physical chemistry must be respected, as was clearly stated by Clark and Lubs, and by Clark alone. The titration of media containing protein substances, meat extract, and inorganic salts, together with the unknown substances which are elaborated by bacteria, is a procedure which can in no way give the true idea of conditions which actually govern the acid-production, or hydrogen-

[19] Jour. Infect. Dis., 1914, 15, p. 227.
[20] Ibid., p. 63.

ion concentration, on account of the buffer effect of the substances present.

An example of the buffer effect in 2 media which were often used in this research is given in the following figures:

I. 1000 c.c. water
5 gm. Witte's peptone
5 gm. K_2HPO_4 — Phenolphthalein acidity 1.0%. Normal
5 gm. glucose

II. 1000 c.c. water — (Chief medium of this research)
10 gm. Witte's peptone — Phenolphthalein acidity 2.1%. Normal
10 gm. glucose
4 gm. Liebig's extract of beef
5 gm. K_2HPO_4

Both of these media are neutral to litmus, and therefore have a hydrogen-ion concentration of about 10^{-7} N. According to Clark and Lubs, the same organism will reach about the same hydrogen-ion concentration in each, and yet the titration figures when these 2 media were inoculated with a laboratory strain of the colon bacillus were: acidity 2.3% normal and acidity 5.4% normal, respectively.

Thus, in cases where the acidity in the 1st medium may show small differences between different cultures, the buffer action of a 2nd medium might be so used as to magnify the differences, instead of affording a more refined method of titration. It is known, however, that titration figures give only relative, and not true values.

In order to determine whether the titration method, as commonly practiced, is the correct one, answers to the following questions were sought:

1. What is the effect of initial acidity on terminal acidity?
2. Shall a blank titration be subtracted from the terminal acidity?
3. What is the effect of surface exposure to air during cultivation?
4. Shall the carbon dioxid be boiled off?
5. Shall the titration be made in a hot or in a cold medium?

INFLUENCE OF INITIAL ON TERMINAL ACIDITY

The fallacy of adjusting the reaction of media has been shown by Clark;[21] but the question arises, "What would be the effect on the growth of an organism of a medium which does not correspond with what we have been pleased to call correct acidity?" If the acidity is shown to be too high it might be expected that either growth could not

[21] Jour. Infect. Dis., 1915, 17, p. 109.

take place or the medium would be so interfered with that the usual results could not be obtained. This point may be further elucidated by determining whether acid-production is the result of growth or of the metabolism of cells in the living state, but which are not actively reproducing.

To study this point 40 c.c. portions of broth containing 1.3% glucose, 0.02% Na_2HPO_4, 0.4% meat extract, and 1.0% peptone were placed in 250 c.c. Erlenmeyer flasks and sterilized. Varying amounts of N/20 HCl were added, the flasks incubated for 24 hours to determine the sterility, and then inoculated with 0.5 c.c. portions of actively fermenting broth cultures of 2 different types. Table 1 is representative of several tests. After the first few tests direct microscopic counts of cells in the various flasks were made, in order to detect a direct relationship between acidity and numbers of bacteria.

TABLE 1

EFFECT OF INITIAL ACIDITY ON TERMINAL ACIDITY AND THE TOTAL NUMBER OF BACTERIA PRESENT

Flask No.	Culture No.	Acid Added c.c. N	Initial Acidity % N	24-Hour Acidity % N	24-Hour Count Cells per c.c.	60-Hour Acidity % N	60-Hour Count Cells per c.c.	120-Hour Acidity % N	120-Hour Count Cells per c.c.
1	*	0.0	1.65	0.73		0.86		1.06	
2	*	2.5	1.83	1.56		1.35		1.41	
3	*	5.0	2.09	1.86		1.84		1.70	
4	*	7.5	2.30	2.17		1.96		1.90	
5	*	10.0	2.56	2.51		2.37		2.40	
6	23	0.0	1.45	1.46	25,500,000	0.55	640,000,000	0.10	47,200,000,000
7	23	2.5	1.51	1.76	58,500,000	1.20	900,000,000	0.10	73,200,000,000
8	23	5.0	1.56	1.81	73,500,000	0.48	1,540,000,000	0.10	74,100,000,000
9	23	7.5	2.15	2.14	58,800,000	0.56	1,000,000,000	0.10	9,570,000,000
10	23	10.0	2.22	2.36	5,180,000	2.03	300,000,000	1.52	7,750,000,000
11	43	0.0	0.80	4.09	36,700,000	4.10	370,000,000	4.30	9,200,000,000
12	43	2.5	1.20	3.58	61,200,000	3.46	440,000,000	3.55	5,680,000,000
13	43	8.0	1.70	†					
14	43	7.5	1.90	3.50	18,800,000	3.40	280,000,000	3.59	4,840,000,000
15	43	10.0	2.43	2.58	4,150,000	2.50	500,000,000	2.52	750,000,000
16	46	0.0	1.97	1.31	62,000,000	0.42	820,000,000	0.30	13,670,000,000
17	46	2.5	0.81	1.86	76,000,000	0.50	550,000,000	0.30	34,400,000,000
18	46	5.0	1.40	2.15	137,000,000	0.65	1,020,000,000	0.40	23,800,000,000
19	46	7.5	2.00	2.25	25,200,000	0.55	474,000,000	0.42	15,300,000,000
20	46	10.0	2.48	2.50	276,000	2.43	124,000,000	2.40	4,300,000,000

* Uninoculated. † Contaminated.

From Table 1 it appears evident that within reasonable limits the initial acidity of a medium does not have a very marked effect on the terminal acidity. Later work has shown that the Organisms 23 and 46 were of the high ratio type of Rogers, Clark, and Davis, while 43 was of the low ratio type. As to the high ratio organisms, it is seen that up to 2% of initial titrable acidity there is no difference in the final acidity. From the rate of growth it is apparent that in the neighborhood of 1.5% acidity the optimum conditions for growth are obtained, in so far as reaction is concerned. That mere numbers of cells do not

play an important part is seen by comparing the numbers of cells in Flasks 8 and 9, and 16 and 17 at the 120-hour period. It is also clear that even in the case of the largest amount of added acid there is actual growth and a reduction of the initial acidity, which might be expected ultimately to reach the same acidity as the corresponding unacidified culture.

With regard to low-ratio Organism 43, it is apparent that within certain limits this organism can reach its maximum acidity within 24 hours, and that after this time the reaction is constant, although growth actually does occur as is shown by the increased number of cells. It is shown further that within these limits of acidity practically the same final acidity has been reached. Too much weight must not be placed, therefore, on small differences of acidity.

From this and similar experiments not recorded here the 1st question may be answered as follows: Within the limits of rapid growth the initial acidity has very little or no effect on the terminal acidity.

SUBTRACTION OF INITIAL FROM FINAL ACIDITY

With reference to Question 2 it may be seen that to subtract the initial apparent acidity from the terminal acidity would be fallacious, and would show nothing of value if the results are to be treated statistically. Brown[22] believed that the amount of acid formed was the vital issue, for he says, "The amount of acid produced by the B. coli group in different carbohydrate media depends in a great part upon the initial reaction . . . The maximum acidity is the amount necessary to prevent further growth." If the amount is the really important point, Brown is correct, for the foregoing data show that the amount of change brought about by an organism is the function of the initial acidity; but when a number of organisms arrive at the same acidity ultimately, and when the problem is approached from different standpoints, it is evident that the common limiting value is the more important and more constant factor.

From Brown's own figures[22] and from Table 1 it may be stated definitely that blank titrations should not be subtracted to determine the acid-production, although this is contrary to the conclusion which Brown himself drew.

The slight change which is observed in the titrable acidity of uninoculated media may be due to the slowness of the buffer action of the medium.

[22] Jour. Infect. Dis., 1914, 15, p. 580.

INFLUENCE OF SURFACE EXPOSURE ON FINAL ACIDITY

For some time we were led to believe that the area of liquid exposed to the air would play an important part in determining the final acidity, but later it was found that these conclusions were erroneous. Subsequent work which is not presented here, owing to the size of the tables involving over 1000 titrations, indicator tests, and Voges and Proskauer reactions, has enabled us to draw the following conclusions, in answer to Question 3: No great difference in the titrable acidity is observed when the bottle (large surface) and deep test tube methods of cultivation are employed. There is in some instances a greater tendency toward variability in the large surface method. The sugar utilization is usually more complete in test tubes than in bottles lying on their sides and exposing 30 sq.c. of surface of liquid.

INFLUENCE OF BOILING BEFORE TITRATION ON THE TITRATION RESULTS AND THE COMPARATIVE VALUES OF HOT AND COLD TITRATIONS

There has always been some discussion among bacteriologists as to the proper methods of determining titrable acidity. The chemist who would titrate a liquid using phenolphthalein as an indicator without first boiling off the dissolved CO_2 would be looked on askance by his associates. But the chemical methods have often been transferred bodily without any question as to applicability, and today nearly all bacteriologists endeavor to get rid of CO_2. Most workers recognize the potential danger of driving off volatile acids or bases by boiling, some attempting to compromise by steaming the tubes, while others continue to boil because it is the official method of the American Public Health Association.

The solution of the problem involves the study of the influence of boiling on both uninoculated and inoculated (incubated) media. Here again it is necessary to determine the most efficient methods of procedure. First, the question arises: Which of the titrations gives the most accurate results, the hot or the cold method? In Table 2 the hot and cold titrations for Clark and Lubs' broth are given. In this test the medium was brought to the boiling point, but not boiled. The flasks marked 'cold' were heated, cooled, and titrated, while the 'hot' flasks were being heated. The heating process took the same length of time in each case.

TABLE 2

ACIDITY OF UNINOCULATED CLARK AND LUBS' BROTH

Titration Number	Titrated Acidity	
	Hot	Cold
1	1.65	1.60
2	1.80	1.55
3	1.70	1.58
4	1.70	1.60
5	1.60	1.60
Average acidity	1.69	1.58

According to Table 2, the hot titration gives higher results than the cold, which fact was observed by Winslow and by Holman,[19] but which has either escaped other observers or has been ignored by them. The more or less popular notion that the hot titration is more accurate as to sensitiveness than the cold titration is disproved in this instance at least.

The higher figure in the hot titration as compared with the cold is probably due to the difference in the degree of ionization of the hot and cold media. The medium when neutral to phenolphthalein while hot becomes pink on cooling and colorless again on subsequent heating. The difference between the hot and cold titrations is probably a measure of the influence of heat on the ionization phenomena in this particular solution.

It may be argued that in making a titration the liquid should be boiled and then cooled. Such a practice would be both time-consuming and erroneous, for it will be shown that the boiling off of CO_2 introduces a very serious error. This is in confirmation of the statement of Anthony and Ekroth.[23]

TABLE 3

A COMPARISON OF HOT AND COLD TITRATIONS OF BOILED CULTURES

Number	Minutes Boiled	Culture Numbers							
		368		G. F. 11		386		1080	
		Hot	Cold	Hot	Cold	Hot	Cold	Hot	Cold
1	0.0	1.16	0.95	2.99	2.81	0.88	0.49	2.28	1.97
2	0.25	1.06	1.07	3.00	2.81	0.92	0.49	2.40	1.97
3	0.5	1.20	0.99	3.00	2.82	0.93	0.68	2.25	1.96
4	1.0	1.24	1.11	2.99	2.76	0.94	0.67	2.25	1.93
5	2.0	1.25	1.30	2.93	2.77	1.06	0.82	2.23	1.98
6	5.0	1.27	1.43	2.94	2.71	1.35	1.14	2.11	1.97

The period of incubation was 5 days.

[23] Jour. Bact., 1916, I, p. 209.

The next point to determine experimentally is whether cultures should be titrated hot or cold. For this purpose several flask cultures were made, samples were taken, diluted, and boiled for the periods indicated. They were done in duplicate, in 2 sets, 1 set being titrated while hot and the other after allowing it to cool to the room temperature. A comparison of the averages is given in Table 3.

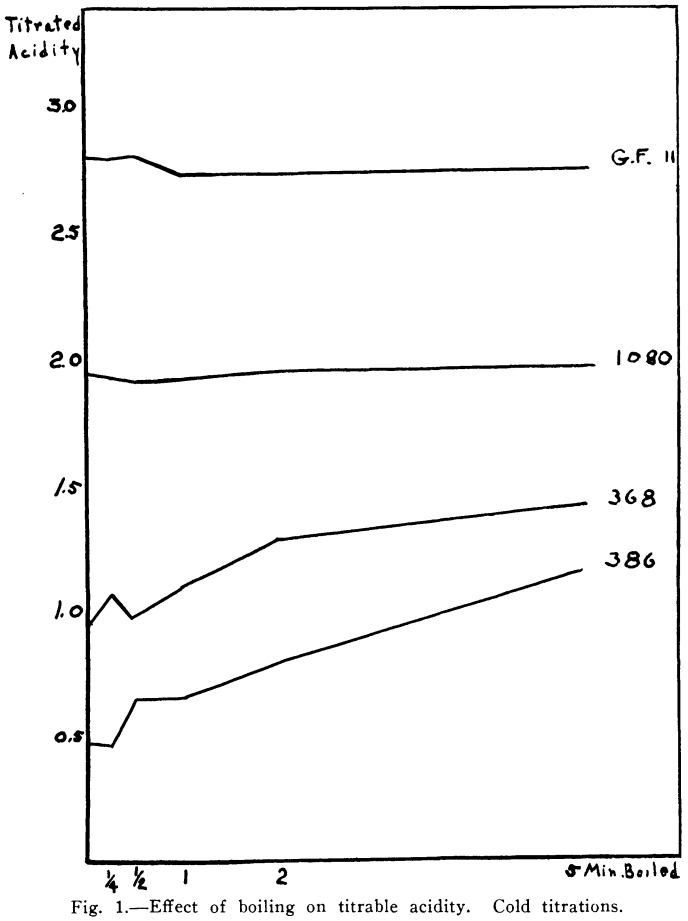


Fig. 1.—Effect of boiling on titrable acidity. Cold titrations.

Table 3 shows that boiled cultures have a tendency to show higher results when hot titrations are employed than when the liquid is allowed to cool to room temperature.

It also shows the effect on the acidity of boiling off CO_2, whether the titration is made hot or cold. This is made more evident graphi-

cally as is shown in Figure 1, where the results of the hot titrations are plotted.

It is difficult to draw any definite conclusions from the results. In general, they seem to show that no CO_2 is removed by boiling, for otherwise one might expect a nearly uniform decrease of acidity for a short time at least. The apparent acidity of the high ratio organisms is increased, due perhaps to the driving off of a volatile base. The general trend of the curve does indicate that boiling or the mere addition of boiling water does not tend to make the results any more constant, but seems to introduce an error in the case of the high ratio cultures, and therefore should not be done. These experiments confirm Clark's statement[21] that the results of titrations are vitiated by the hot method.

On the other hand, the recent abstract of Noyes[24] stating that CO_2 in distilled water will introduce grave errors cannot be accepted in the sense that he seems to have meant it. He says that distilled water or double-distilled water does not mean carbon-dioxid-free water, and further, "CO_2 in distilled water makes it possible to have a medium titrate plus 1.0% when it is really neutral or alkaline." It seems as though he must have been working with distilled water particularly rich in CO_2 to arrive at such conclusions, but as his data have not yet been pulbished, this is only a conjecture.

On completion of this preliminary work it was decided to cultivate the organisms in glucose-phosphate broth (the composition of which has been given), in bottles and in test tubes. The titrations were made in the cold, without boiling off CO_2, the culture fluid being diluted with cold distilled water which had been recently boiled. The period of incubation was 5 days at 37 C.

BIOMETRIC STUDIES OF THE GAS-FORMERS OCCURRING IN NATURE

Preparation of Cultures.—About 1000 samples of soils, leaves, twigs, flowers, bark of trees, berries, sand, snow, etc., were collected in places which were situated as far as possible from all sources of pollution. These samples were taken from isolated fields in Illinois, sand dunes in northern Indiana, mountain tops in Connecticut, and the uppermost points of the watershed of the New Haven Water Company. The possibility of fecal contamination on the watershed

[24] Jour. Bact., 1916, 1, p. 87.

and on the mountain tops is extremely remote. With very few exceptions there was no reason to believe that there was any recent fecal contamination of any of the samples from the Middle West. The exceptions mentioned and indeed any samples which were taken from places where the drainage was toward the point of sampling, are treated separately, thus necessitating 2 groups of bacteria, 1 set being designated as 'O. K.' from the sanitary standpoint, and the other marked 'Discards.' The classification study was made on the combined sets.

All of the samples were inoculated in glucose broth and incubated at 37 C. for 3 days. Any of the cultures which showed gas-production were plated. The plating was done originally on litmus glucose agar, but during the 2nd year it was found that the predominating gas-former in soil was not an acid-producer in these agar plates. The use of litmus was discontinued and it was found necessary to test the gas-production of representative agar colonies. Gas-forming cultures were plated out again and isolated colonies tested for gas-production, as before. This process was carried out a 3rd time, after which a well isolated colony was selected for the inoculation of the medium whose composition is given on p. 165 (II). Following an incubation period of 5 days at 37 C., the broth cultures were titrated.

Whenever there was any question as to the purity of any given culture the following method of replating was carried out: The culture material was vigorously shaken for 5 minutes in a large test tube containing broken glass and 5 c.c. of water, after which a loopful of the emulsion was transferred to a 2nd tube and the shaking repeated. Plates were poured from the 2nd tube. Glucose broth in Durham fermentation tubes was inoculated from 5 of the most varied colonies.

The morphology of the organism was studied on Gram-stained slides. With few exceptions the gas-producing organisms were Gram-negative, short rods having spores were never observed. In their microscopic appearances and in their general behavior they resembled the colon bacillus. The Gram-positive organisms that were met with only occasionally were spore-producers and relatively large, and it was evident that they were members of a widely different group.

Biometric Data.—Cultures were made in the glucose phosphate broth (II, p. 165) in Durham fermentation tubes and in 4-ounce glass bottles which gave a surface exposure of 30 sq.c. when laid on their sides. Approximately the same amount of liquid was placed in the tubes as in the bottles.

Titrations were made as indicated.

Methyl red was added to the broth to determine on which side of the methyl red neutrality point the acidity lay.

Qualitatively sugar determinations were made at first with Fehling's solution, but a purple color was observed to develop in many cases which was in all probability due to the Voges and Proskauer[25] reaction, brought about by boiling with the strongly alkaline solution. Consequently Benedict's solution (2nd modification) was substituted and the troublesome purple color did not appear. Saccharose was inverted by boiling with a few drops of sulphuric acid for a half hour.

[25] Ztschcr. f. Hyg., u. Infektionskrankh., 1898, 28, p. 20.

The value of the Voges and Proskauer reaction in its relation to this group of organisms was not appreciated until about half of them had been studied. It was tried with all of the sugar media and with some of the glycerin broth tubes. In glycerin media the reaction was present in a great many instances, but it was weak and uncertain and therefore discontinued. The reaction was obtained by adding 10 c.c. of 2-4% KOH to the sugar media and letting them stand over night.

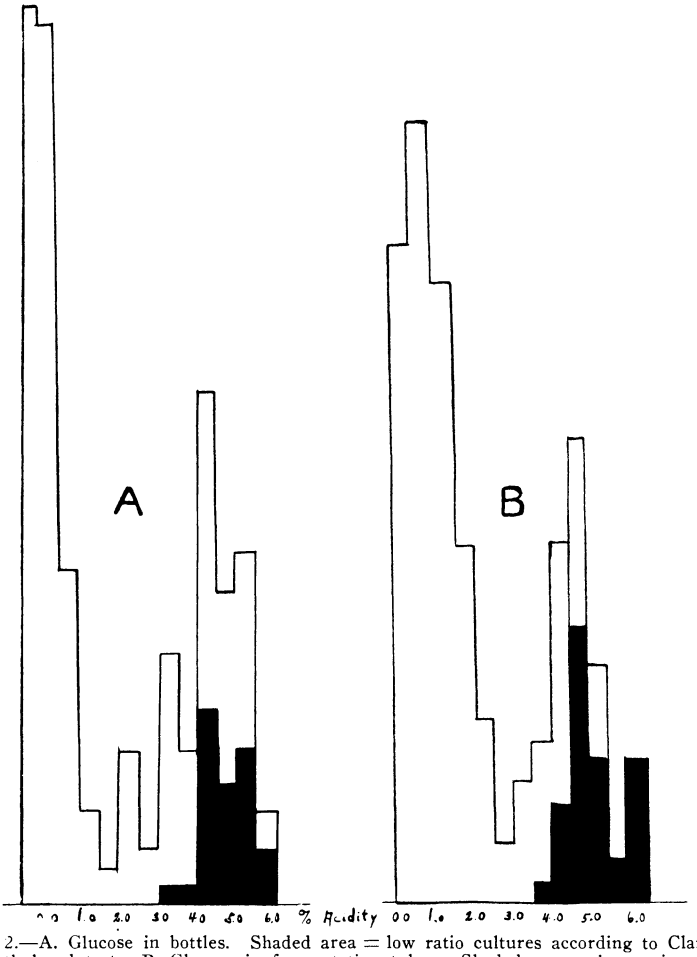


Fig. 2.—A. Glucose in bottles. Shaded area = low ratio cultures according to Clark and Lubs' methyl red test. B. Glucose in fermentation tubes. Shaded area = low ratio cultures according to Clark and Lubs' test.

Pigment-production was recorded in the standard color terms of the chart of the Society of American Bacteriologists. Owing to the large number of cultures and to the vast number of other tests, it was impossible to go into the subject of pigment-production as extensively as Rogers, Clark, and Evans.

The determination of gelatin liquefaction by the standard method was undertaken, but this procedure was abandoned as too uncertain, and the method proposed by MacConkey[2] was tried and found to be more satisfactory. This test is made by using 5% gelatin and incubating at 37 C., removing the tubes from time to time and cooling for several hours. In this way all liquefiers were

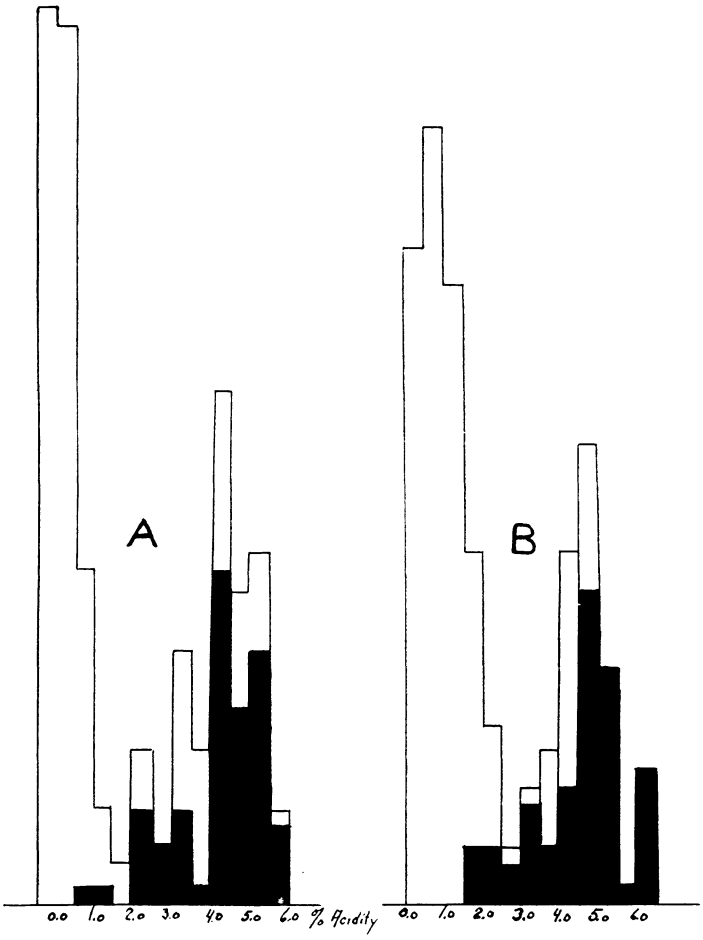


Fig. 3.—A. Frequency curve. Glucose in bottles. B. Frequency curve. Glucose in fermentation tubes. Shaded area = organisms producing gas in glycerol.

detected in 10 days. The advantage of obtaining data in less than 2 weeks instead of waiting 2 months need not be dwelt on.

Nitrate-reduction tests were made according to standard methods.

Indol determinations were made by Toby's method[26] after 8 days of incubation in Dunham's broth at 37 C.

[26] Jour. Med. Research, 1906, 15, p. 301.

Starch hydrolysis was followed by streaking starch plates and incubating them 1 week at 37 C. Iodin in KI solution was added to determine the disappearance of starch.

Spore tests were conducted on organisms by heating in glucose phosphate broth in Durham tubes at 80 C. for 15 minutes, using known colon bacillus as a control.

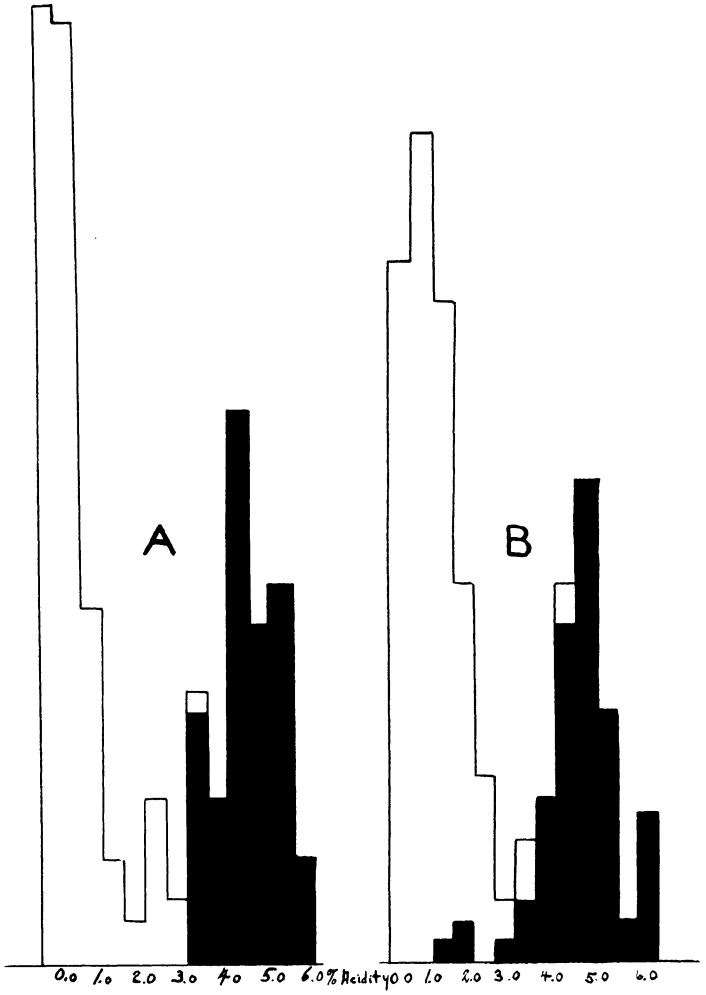


Fig. 4.—A. Glucose in bottles. Shaded area = organisms making sufficient acidity to show acid reaction of methyl red in bottle cultures in Medium II. B. Glucose in fermentation tubes. Shaded area = organisms making sufficient acidity to show acid reaction of methyl red in fermentation tubes in Medium II.

Frequency curves were plotted from the data obtained in the 2 sets of titrations of the glucose media (in test tubes and in bottles). The curves are reproduced in Figure 2. It can be seen that both have the

same general contour, and that both exhibit 2 modes; but, although it cannot be shown in curves of this size, the organisms in the low acid mode of 1 curve are not always in the low acid mode of the other

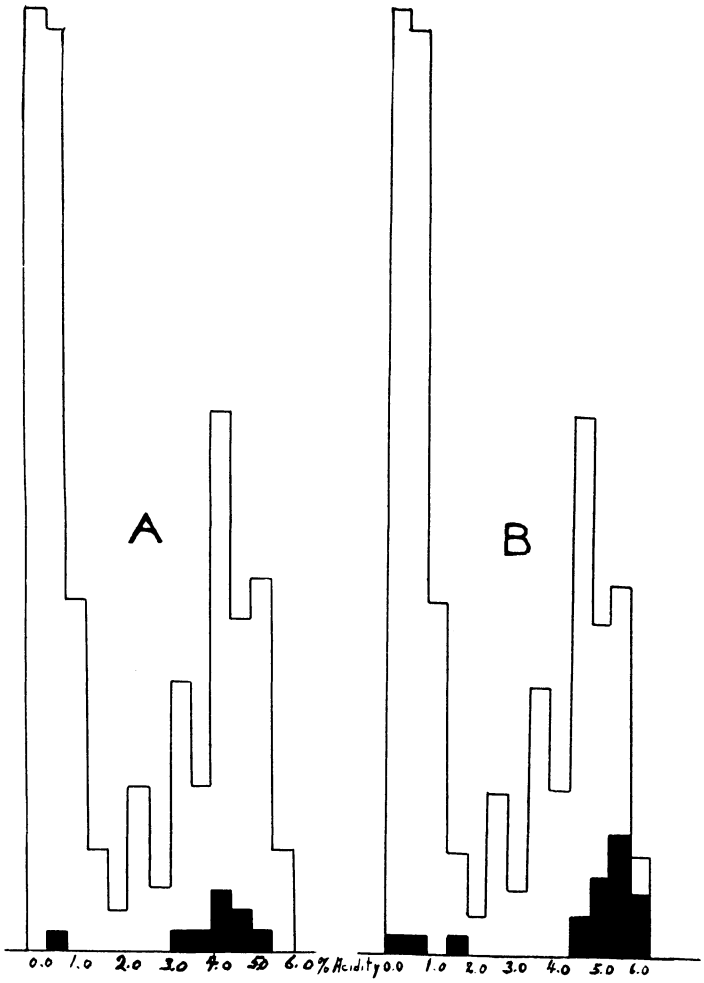


Fig. 5.—A. Glucose in bottles. Shaded area = organisms failing to ferment lactose with gas-production. B. Glucose in bottles. Shaded area = organisms failing to ferment saccharose with gas-production.

curve. An extended study of this point was made and the discrepancy ascribed to variability of acid-production. This subject will be taken up under the discussion of Stability and Variability of Characters. From all that we have been able to observe we are led to conclude

that the low ratio organisms remain quite constant, but that the high ratio strains produce small or large amounts of acid without any apparent law governing the phenomenon. This is shown in Figure 2 where the shaded area gives the location of the low ratio cultures in the frequency curves. It will be seen that they are confined to the high acid modes.

In seeking an explanation of this peculiarity of the high ratio organisms, contamination and differences in the degree of aeration may be excluded, for it was observed again and again under a variety of conditions. It will be shown later that the brand of peptone employed has a great deal to do with the variability. In all of the work described herein Eimer and Amend peptone was used, except where otherwise noted.

Figure 3 shows the correlation of the glycerol fermenters and the high acid mode. It is evident that the glycerol fermenters do not vary as much as do the nonglycerol fermenters.

Figure 4 shows the positions of the organisms which make sufficient acidity to give the acid reaction of methyl red when grown in the glucose-phosphate (II, p. 165) medium. This reaction should not be confused with the methyl red test of Clark and Lubs.* It shows that when the organisms vary in the direction of high acid-production the hydrogen-ion concentration runs quite high, and the organisms begin to assume the characters of the low ratio type.

A few of the organisms failed to ferment both saccharose and lactose, although none failed to ferment one or the other of these sugars. The results are shown in Figure 5.

The Voges and Proskauer reaction is given by all methyl red negative organisms when the acid-production places them in the low acid mode, but when the acid-production runs high enough to place them in the high acid mode the Voges and Proskauer reaction may or may not be present. A possible reason for this will be apparent in the discussion of the mechanism of the variability. When employing the methyl red test in the prescribed manner of Clark and Lubs, that is, using 0.5% Witte's peptone, 0.5% glucose, and 0.5% K_2HPO_4, the Voges and Proskauer reaction given is always in the methyl red negative cultures. Occasionally there have been cultures in which the acid-

* Clark and Lubs' methyl red test is made by inoculating a special medium whose composition has been previously stated, and incubating 5 days at 30 C. Two drops of methyl red solution are added to determine the reaction toward this indicator. Our use of methyl red, in addition to the bona fide Clark and Lubs method, was the addition of a few drops of it to our special medium (p. 5) to determine its reaction toward this indicator.

production was sufficient in our medium to give an acid reaction with methyl red and a positive Voges and Proskauer reaction.

The saccharose and lactose curves show 2 modes, but little is to be gained from a study of them.

Summary.—1. The frequency curve for the glucose fermentation, whether cultivation be carried out in fermentation tubes with little aeration or in bottles with much surface exposure, is approximately the same and shows 2 very distinct modes.

2. The methyl-red-positive organisms are to be found always in the high acid mode.

3. Certain of the methyl-red negatives produce sufficient acid in our medium (II, p. 4) to show the acid reaction of methyl red, and may or may not possess other supposedly correlated characters.

4. The organisms which ferment glycerin with gas-production seem to show a lesser tendency toward variability than do the non-fermenters.

5. There is little to be gained from a statistical study of the saccharose or lactose fermentations of this particular group of organisms.

6. Variation in acid-production occurs to a sufficient extent to cause some doubt as to whether the biometric method is of service in the study of this group of bacteria.

STABILITY AND VARIABILITY OF CHARACTERS

Variability is a term which is often inaccurately used. Strictly speaking, it means the temporary alteration of 1 or more characters, as contrasted with mutation, which implies permanent change.

Revis,[27] being of the opinion that the colon bacillus was originally a natural soil organism, believed that there should be a loss of certain functions when this organism is transferred to a new habitat, the intestine of man, in accordance with the usual behavior of parasites, and that, when returned to its original abode, it ought perhaps to regain some of its lost characters. Experiments to prove this with the colon bacillus failed, while those with B. [lactis] aerogenes seemed to give somewhat conflicting results. Continued cultivation of B. [lactis] aerogenes on synthetic media caused it to lose some of its characters apparently, such as the Voges and Proskauer reaction and the power of fermenting saccharose. This occurred in 2 instances, and the organisms became suspiciously similar to the colon bacillus, "the loss of the Voges and Proskauer reaction constituting a fundamental change."

[27] Centralbl. f. Bakteriol., 1910, ii, Ref., 26, p. 161.

Rogers, Clark, and Evans[7] expressed the belief that the study of the Voges and Proskauer reaction would clear certain difficulties in the study of the colon group. This reaction, however, is not always constant and is not always correlated with other factors, as was expected by some workers. For instance, MacConkey[28] believed that a positive Voges and Proskaur reaction should be correlated with a definite gas ratio in the colon-aerogenes group, but frequently found it positive when the gas ratio was not characteristic. On the other hand, there was no tendency of the colon bacillus to alter its characters.

In 1909 he reported further on the variability of the Voges and Proskauer reaction and found in a few cases that it varies inversely with the indol-production, and to such an extent that he doubts the value of the reaction as a diagnostic measure. All of the Voges and Proskauer variants possessed the same fermentation reactions.

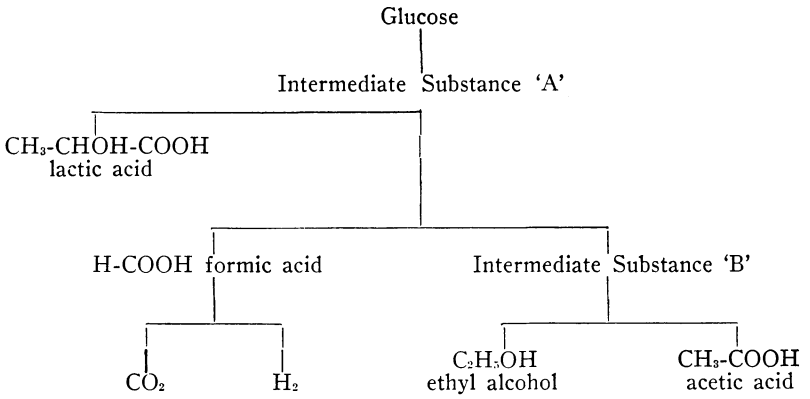
Levine has shown[10] that the Voges and Proskauer reaction is correlated with the methyl red negatives, while Johnson[18] found that not all of the presumably high ratio group were able to produce a positive Voges and Proskauer reaction. These observations are quite suggestive, in interpreting the work of others. Rogers, Clark, and Davis[6] and Rogers, Clark, and Evans[8] noted a constancy in the amount of gas-production and of gas ratio in low ratio organisms, while with regard to the high ratio group they repeatedly comment on its variability. They also found instances where an organism had apparently altered its gas ratio quite profoundly, but state that there may have been an error in lettering the cultures. This is undoubtedly the safer explanation, but in view of the results of others in this field, and of the results of our own investigation, the question of variability appears highly important.

Harden and his associates[29] have shown the Voges and Proskauer reaction to be due to a special decomposition of glucose by several organisms, chief among which are B. cloacae and B. [lactis] aerogenes, with the formation of large amounts of CO_2 and small quantities of hydrogen, together with a substance known as 2-3 butanediol, or butylene glycol, CH_3-CHOH-CHOH-CH_3. They further showed that in the presence of oxygen the organisms have the property of oxidizing the 2-3 butanediol to acetyl-methyl-carbinol, CH_3CO-CHOH-CH_3 which is the substance that gives the Voges and Proskauer reaction. When the acetyl-methyl-carbinol is left in contact with air in the presence of strong alkali it undergoes oxidation to diacetyl, CH_3-CO-CO-CH_3, and if peptone is brought in contact with the diacetyl in the alkaline solution a pink compound is formed which usually possesses a greenish fluorescence. More recent work by the same investigators has tended to show that certain amino-acids are the agents in the peptone solution which have to do with the formation of the characteristic pink color of the Voges and Proskauer reaction.

Thompson[30] studied the chemical action of B. cloacae on glucose and mannitol, using B. [lactis] aerogenes and the colon bacillus for comparison. Some of his data like those of Harden are of particular interest in theorizing on the variability in this group. According to this investigator there is a marked resemblance between B. cloacae and B. [lactis] aerogenes.

Grey's theory[31] of the decomposition of glucose by the colon bacillus is graphically represented as follows:

[28] Jour. Hyg., 1905, 5, p. 333.
[29] Jour. Physiol., 1911, p. 332. Proc. Roy. Soc., 1911, B 84, p. 492.
[30] Proc. Roy. Soc., 1911, B 84, p. 500.
[31] Proc. Roy. Soc., 1914, B 87, p. 472.



In the present problem the foregoing facts taken from the literature have led to the formulation of a theory which may serve to explain the inner metabolism of the 2 types of organisms, and incidentally to explain the mechanism of the variability. The theory is based on the following observations:

1. The low ratio organisms do not vary to any appreciable extent. They remain consistently Voges and Proskauer negative, and methyl red positive. They do not utilize all of the sugar present in the culture media, and the titrable acidity is always high, depending of course on the extent of the buffer action of the medium.

2. The high ratio organisms vary apparently without law, in several respects:

(a) While they ordinarily produce little or no acid, there are times when they produce high acid, with a sufficiently high hydrogen-ion concentration to react acid to methyl red. When using Witte's peptone in the prescribed methyl red test the variability in this respect is small, and is confined chiefly to the yellow pigmented forms. But in certain American brands of peptone that were tried, the variability was so great that the assertion of Clark and Lubs that no other peptones than Witte's can be used is substantiated to a certain extent. This has led to a study of the influence of different peptones on the methyl red test and on variability, which will be presented later.

(b) In addition to the variability in acid-production, there is variability in the amount of sugar used, and this variation is always parallel to the acid variation. Kendall[4] says, "Members of the cloacae group decompose glucose rapidly. Even at the end of 24 hours the sugar has disappeared from the culture medium, as is shown by actual sugar

determinations. The organisms therefore attack the protein and this explains the large amount of ammonia produced in glucose broth after the first day . . . . This is an important and permanent distinction between the colon and cloacae groups." In spite of Kendall's statement regarding the permanency of this point, it has been our experience that whenever the organisms of the cloacae group vary in the direction of high acid-production, the sugar utilization is never complete, as it so frequently is when the fermentation proceeds in what might be termed the normal manner for the cloacae group. There is a very definite variation in the amount of sugar used by the organism, depending on whether there is acid variation or not. When the acidity runs high very little sugar is used, and when the acidity remains low the sugar utilization is complete or very nearly so, depending on several factors, chief of which is the kind of peptone used and the length of time of incubation.

(c) When the variation from low to high acid occurs there is a tendency for the Voges and Proskauer reaction to disappear, and frequently when the acidity is high enough to show the acid reaction of methyl red in any sort of medium, either our glucose-phosphate broth, or Clark and Lubs' broth, the Voges and Proskauer reaction is extremely weak or totally absent. This might be ascribed to contamination, but frequent replating and selection of different colonies fail to give a constantly high acid-producing line in addition to the normal organisms. Furthermore, an organism will on one day behave in the usually accepted manner, that is, low acidity and complete sugar utilization, and on another it will vary in the direction of high acid-production, after which its behavior will be normal again.

The Voges and Proskauer reaction seems to be inseparably locked with the sugar utilization; hence, where little sugar is used the reaction is generally negative, or in doubt because of its faintness.

(d) When the acidity runs high and the sugar utilization is small, and when the Voges and Proskauer reaction is very weak or absent entirely, the amount of gas produced from glucose in the closed arm of the Durham fermentation tubes is not around 100%, as is usually the case, but is in the neighborhood of 50%, and as near as can be determined by this crude method the CO_2 to H_2 ratio is about 1 : 1.

Rogers and his associates have frequently suggested that there seem to be 2 parallel fermentations going on, one of which produces CO_2 in great preponderance over H, while the other produces approx-

imately equal parts of H and CO_2. They believe that the 2 reactions are mutually dependent, but the whole problem of variability in this group would be easily solved if it were shown that they are independent.

(e) The last important point in formulating the new theory is that whenever there is a variation toward high acid-production there is little growth in glucose media, and after a day or 2 the organisms begin to sediment out leaving a comparatively clear liquid which is quite comparable to the appearance of a glucose broth culture of the colon bacillus; whereas, B. cloacae when fermenting normally will give an extremely cloudy broth culture, which never clears.

If there are 2 reactions in the cloacae group, one of which is quite similar to the colon fermentation and the other to the 2-3 butanediol fermentation, as suggested above, then it is possible to explain the variability of this group with respect to amount and kind of gas-production, the titrable acidity, the hydrogen-ion concentration, the methyl red rest which is dependent on it, the Voges and Proskauer reaction, the sugar utilization, and the amount of growth, providing the 2 reactions are considered independent to a certain degree.

Of course, contamination could explain the variability, but if a contaminating organism were present it would have to be one of the colon group, and a high acid former, and always the same organism. We appreciate the difficulty of excluding contamination, but it has never been our experience to meet the same contaminating organism in as many as 150 cultures. Again, the contamination would have to occur in the high ratio cultures only to be manifest. And lastly, contaminating organisms have been sought repeatedly and never found, except in 2 instances.

Of the 2 possible explanations the contamination idea seems to be the less likely, especially as MacConkey[28] has shown that B. cloacae grown in the presence of the colon bacillus will show the Voges and Proskauer reaction, and in many of our cases of variation the Voges and Proskauer reaction disappears entirely.

As far as is ascertainable from the data at hand there is no apparent rule which governs the variability, but it seems to be a matter of chance as to which fermentation will predominate. Assuming that the CO_2-2-3 butanediol fermentation predominates and that the colon-like fermentation is subordinated, as is apparently the case in the 'normal' B. cloacae sugar decomposition, in accordance with the theory

that the 2 fermentations are independent, the following conditions will prevail in the glucose phosphate broth culture:

The fermentation will proceed with increasing rapidity until the sugar is all gone, because the end-products of the predominating type of fermentation are essentially nonacid in character and the 2-3 butanediol, according to Thompson, is a food for B. cloacae. The colon-like fermentation will proceed to a slight extent, and a very small amount of acid will be formed together with equal parts of CO_2 and H. Following the loss of all the sugar there will occur more or less 'protein decomposition' depending on its availability, and thus a certain amount of ammonia-production will take place which would account for the alkalinity of some cultures.

2. There will be a very heavy growth in the tube, because of the absence of end-products of acid character to act as inhibitors, and because, as has been mentioned, the butanediol can be used further as a food.

3. A very large volume of gas will be formed, because the gaseous fermentation will not be stopped by anything other than the complete exhaustion of the sugar.

4. The gas ratio will be 'high', in the sense of Rogers and his associates, consisting of 2-4 parts CO_2 and 1 part H.

5. The presence of the 2-3 butanediol will give rise, after bacterial oxidation, to acetyl-methyl-carbinol, which in turn will be responsible for the positive Voges and Proskauer reaction of this culture.

6. The acid-production being small, the subsequent alkali-production will permit of a very small hydrogen-ion concentration only, and if methyl red is added to the culture it will show an alkaline reaction.

A large volume of gas, high gas ratio, and low acidity seem to be inseparable.

On the other hand, let us consider what will be the situation resulting from the reverse of the previous conditions. In this case the colon-like fermentation will predominate and the CO_2-butanediol fermentation will be subordinated. For the time being, the cause of this reversal will not be considered. Step by step the following conditions will be found:

1. The fermentation will proceed until the acid produced from the colon-like fermentation will inhibit the growth, and practically no other change will take place in the culture. Only a small fraction of the sugar in the medium will be used. All reactions which may occur

will have to take place in the time elapsing between the time of inoculation and the time when the inhibiting acidity is reached.

2. If left in this condition for a few days most of the organisms will settle to the bottom of the tube, thus leaving a clear solution which will approach that of a culture of the colon bacillus under similar conditions.

3. Because of the rapidity with which the colon-like fermentation reaches the inhibiting acidity, or because the causal agent of the variation has slowed down the CO_2-butanediol fermentation, little CO_2 will be formed by this method, and the volume of gas will therefore be small, approximating the volume expected from the colon bacillus under similar conditions.

4. The gas will approximate equal volumes of H and CO_2, with the latter probably slightly predominating, and the organisms will approach those of low ratio.

5. Because of the slowness or the lack of the CO_2-butanediol fermentation there will be few or none of the substances formed which go to make a positive Voges and Proskauer reaction and consequently the reaction will be negative.

6. The acid-production being high the cultures will give the acid reaction of methyl red.

A small volume of gas, low gas ratio, and high acidity seem to be inseparable.

A 3rd condition must be taken into account, namely, the failure of either fermentation to predominate. In such an event, it would be possible to have a certain amount of 2-3 butanediol formed before the inhibiting acidity is reached and would result in the paradoxic condition of an organism being simultaneously methyl red positive and Voges and Proskauer positive. The gas ratio would be lower than would be the case in the 1st of the foregoing assumed conditions, and higher than in the 2nd.

In the foregoing theoretical discussion reference is made to media whose composition was given on p. 165, and made of peptone other than Witte's. In Clark and Lubs' broth containing American peptones instead of the Witte all of the conditions do not hold, and it is suspected that this is due, at least in part, to the higher amino-acid content of the American brands as compared with Witte's peptone.

Table 4 shows the manner in which acidity varied and led to the study of the cause of variability. It is a collection of data taken from

cultures of the same strains at different times, except that those which are in the column headed 4/19/16 are from cultures which have been plated out for the purpose of once more detecting possible contamination.

TABLE 4

A COLLECTION OF TITRATION FIGURES SHOWING VARIABILITY IN ACID-PRODUCTION

Number	Acidity Expressed as % Normal				
	3/9/16	4/1/16	4/4/16	4/12/16	4/19/16
6a contaminated	3.4	3.9	...	—0.5	...
6b	0.3	4.7	...	—0.3	...
7a	3.8	4.8	...	4.7	...
7b	3.2	4.8	. .	4.5	...
9a	0.0	0.0	...	1.3	...
9b	0.0	0.0	. .	3.9	...
11a P.	3.4	4.5	...	3.2	...
11b P.	3.8	5.0	...	2.6	...
12a	0.0	4.5	...	—0.4	...
12b	0.0	4.5	...	—0.3	...
13a	3.3	3.9	...	—0.4	...
13b	3.3	0.0	...	0.0	...
14a	4.2	4.0	...	5.3	...
14b	4.4	6.0	...	0.2	...
15a	4.1	6.7	...	—0.2	...
15b	4.2	7.0	...	0.2	...
16a	4.1	6.2	...	—0.2	...
16b	3.2	0.0	...	—0.1	...
22a	0.5	—0.3	...	4.1	...
22b	0.4	—0.7	...	3.4	...
23a	0.3	—0.3	...	2.9	...
23b	0.0	—0.4	...	2.4	...
24a	—0.2	0.1	...	2.4	...
24b	—0.2	0.2	...	0.0	...
29a	5.6	3.5	5.2	...	...
29b	4.2	3.9	4.1	...	...
31a	0.3	0.1	3.8	—0.2	...
31b	4.6	...	3.3	—0.4	...
32a	4.0	...	0.3	—0.3	...
32b	3.7	...	0.2	0.0	...
33a	3.3	...	0.2	—0.2	...
33b	4.2	...	0.0	—0.4	...
34a	4.0	...	0.0	—0.4	...
34b	2.6	...	0.0	—0.3	...
35a	0.0	...	3.6	—0.2	...
35b	0.0	...	3.8	—0.5	...
36a	0.1	...	4.3	—0.3	...
36b	3.8	...	0.0	0.0	...
37a	4.3	...	0.5	—0.4	...
37b	4.4	...	2.3	—0.3	...
38a	3.6	...	3.1	—0.4	—0.2
38b	0.8	...	3.8	—0.3	3.6
39a	—0.2	...	3.9	—0.5	4.2
39b	0.0	...	3.8	—0.3	4.4
40a	0.0	...	3.8	—0.3	4.4
40b	0.0	...	3.7	0.0	4.2
41a	0.0	...	0.0	...	4.3
41b	—0.2	...	—0.3	...	4.3
42a	—0.4	...	0.0	...	—0.5
45a P.	2.0	...	4.4	4.0	...
45c P.	4.5	...	4.2	...	4.5
46a P.	4.8	...	4.2	...	0.5
46b P.	4.8	...	4.7	...	3.9
47a P.	5.4	...	4.6	...	1.6
47b P.	5.0	...	4.4	...	4.8
49a	—0.2	...	4.1	0.0	1.3
49b	—0.2	...	3.9	0.1	0.6
50a	0.0	...	4.3	0.0	0.0

P = pigment formers.

In Table 4 all of the organisms reported are presumably of the high ratio type, as was shown by Clark and Lubs' methyl red test. It will be noted that there is no apparent uniformity of variation, and parallel strains such as the 'a' and 'b' strains of a given number are not always subject to the same direction of variation at the same time. With the exception of 6a which was 1 of the 2 cultures that had been found contaminated, and the pigmented forms 11, 45, and 47, all have given reason to believe that they are high ratio organisms, but the pigmented forms behave in such a peculiar manner that it is impossible to state definitely whether or not they are high ratio also. The fact that they occasionally give the Voges and Proskauer reaction suggests the high ratio classification.

It may be seen why the biometric method in the classification of this group will be of little service. If data cannot be duplicated from time to time within the limits of very slight variability no system of classification can be based on these characters.

When an organism in this group varies it will be seen that the change is usually quite profound, and that there are few which might be called half variants. A culture is usually at one extreme or the other. This is further evidenced by the sharply defined modes of the frequency curves. Very few cultures are to be found in the intermodal space, while a few of the organisms which are found in the high acid mode would be expected to be in the low acid mode from their general method of metabolism, such as the presence of the Voges and Proskauer reaction.

If the 2 combinations of characters mentioned, namely, large volume of gas, high gas ratio and low acidity, on the one hand, and small volume of gas, low gas ratio, and high acidity, on the other, can be proved to be absolutely interlocked, as seems quite possible, then the correlations observed by Clark and Lubs which find expression in their methyl red test are easily explained. It can be seen that of necessity the methyl red positives gave a low gas ratio and vice versa; and further, that the correlation of methyl red negatives with Voges and Proskauer positives, as noted by Levine,[10] must necessarily follow.

Attempts were made to secure permanent mutants by fixing the variant in the high acid type of metabolism, in order to determine whether or not other characters varied with the glucose fermentation. All efforts of this nature were unsuccessful. It was thought that perhaps the colon bacillus might arise from B. cloacae or B. [lactis]

aerogenes when these are taken into the animal intestine, but cultivation of B. cloacae in the presence of bile at 37 C. during several transfers and then plating out did not result in the acquisition of any new characters, although there was evidence that the CO_2=2-3 butanediol fermentation was somewhat restrained by this method of treatment, in comparison with the gas-production of the colon bacillus controls

Cultivation in liquid Endo's medium or in broth containing Gentian violet, paranitrophenol, brilliant green or methyl red, was also unsuccessful. It may be of interest to note that the methyl red seemed to exert the greatest inhibition of all of the substances tried.

INFLUENCE OF DIFFERENT PEPTONES ON THE METHYL RED TEST

It has been stated by Clark and Lubs that no peptone other than Witte's would serve for the methyl red test, because in using other brands the results were inconsistent. If the results with other brands of peptone are inconsistent, the cause of this inconsistency must be some form of variability. The fact that all of our earlier studies were made with Eimer and Amend peptone, using meat extract and varying degrees of aeration, led to the suspicion that the variability might be a function of the medium as well as of the organisms. A set of experiments was planned which should elucidate the following points:

1. Is variability an inherent property of the cell or is it impressed by some outside forces?
2. What is the influence of one kind of peptone as compared with another?
3. What is the influence of meat extract?
4. What is the influence of aeration on the methyl red test?

To determine these points it seemed wisest to use a simple medium which would combine as many tests in a single series as possible. Such a medium was found in Clark and Lubs' broth, by substituting one peptone for another and by the addition of meat extract. Owing to a shortage of pure K_2HPO_4, the corresponding sodium salt was substituted, using the stoicheiometrical equivalent, and in a series of tests comparing the 2 salts it was found that the methyl red tests gave identical results whether the potassium or sodium phosphate was employed.

Witte, Eimer and Amend, Armour, and Digestive Ferments Co. peptones were used.

Each brand of peptone was made up into Clark and Lubs broth and divided into 2 portions. To 1 part 4 gm. per liter of Liebig's meat extract were added. Both lots were then placed in test tubes and in the previously mentioned 4-ounce

glass bottles. The tubes and bottles were sterilized in the autoclave after a series of tests had been made to determine whether the medium containing Witte's peptone could be used with the same results when it had been autoclaved as when it had been sterilized intermittently. The results on 50 tests were identical. In each series of tests the following determinations were made: titrable acidity, methyl red reaction, presence of residual sugar, Voges and Proskauer reaction, and in a few instances the Sörensen titration.

Lastly a series of tests was conducted using meat extract alone in the place of peptone, and another using neither meat extract nor peptone, but Uschinsky's medium minus the glycerol.

As these experiments required about 1200 cultures the incubation time was cut from 5 to 3 days and the temperature raised from 30 to 37 C. Levine[10] recommends this change also. It was found that with respect to the methyl red test alone the results under the new temperature conditions were identical with those obtained by the prescribed method.*

Summary. — The medium may in a small measure increase or decrease the variability of the organism, but the fundamental cause of variability is associated with the property of the high ratio cultures to bring about 2 parallel methods of sugar decomposition which are more or less independent.

When using Witte's peptone without meat extract the sugar utilization was never complete, and even when meat extract was added to the medium it was in many instances incomplete. The amount of sugar utilization was greater in the test tubes than by the bottle culture method. These results are quite in contrast to those obtained with Eimer and Amend peptone, in which in many instances the sugar completely disappeared without the aid of the meat extract. In the medium described on p. 165, sugar decomposition was even more often complete. This may have been due, however, to the fact that 1.0% peptone was employed instead of 0.5%.

There is, nevertheless, a marked difference in the action of different peptones, and it is to be inferred that this is referable to the amino-acid content, in amount or kind, of the peptones. This inference is strongly supported by Table 5, in which Witte's peptone is seen to contain the smallest amounts of amino-acids, as indicated by the Sörensen titration. It is with this peptone that the least variability has been observed.

In the modified Uschinsky medium without glycerol, but containing 0.5% K_2HPO_4 and 0.5% glucose, there was little or no growth of the low ratio organisms, while the high ratio strains grew vigorously. Sugar utilization by the high ratio organisms was almost complete, and there is reason to believe that if sufficient time were allowed, all

* Owing to their size the tables are not reproduced here. They may be found, however, in the unabridged thesis in the Yale Library, or duplicate copies thereof held by the authors.

TABLE 5

COMPARISON OF SORENSEN TITRATIONS OF CLARK AND LUBS' MEDIUM MADE WITH DIFFERENT PEPTONE

	Without Meat Extract		With Meat Extract		
Witte Peptone					
NH_3	0.80	0.81	1.4	1.2	1.54
Amino-acids	0.55	0.60	0.6	0.5	0.61
Eimer and Amend Peptone					
NH_3	0.75	0.85	1.3	1.3	
Amino-acids	0.86	0.89	1.10	1.22	
Dif Co. Peptone					
NH_3	0.70	0.70	1.11	1.11	
Amino-acids	0.65	0.63	0.99	1.00	
Armour Peptone					
NH_3	0.74	0.73	1.13*	1.20	
Amino-acids	0.99	0.91	1.21	1.30	

* Average of 6 titrations on another lot of the same medium.

members of this group would entirely dispose of the sugar. The methyl red reaction is meaningless in this medium. On the other hand, it is of particular interest to note that the high ratio organisms are able to produce the necessary conditions for a positive Voges and Proskauer reaction, in spite of the fact that there is no added protein.

In the light of the foregoing biometric studies it may be said that biometry is of little or no value in the face of unrestricted variability. Unless one or the other of the 2 important characters of the fermenting organisms of this group, preferably the colon-like fermentation of the high ratio members, can be restrained, efforts to obtain dependable results will be unsuccessful.

Conclusions as to the Influence of Medium and Methods of Cultivation.—1. Variability must be reckoned with.

2. Witte's peptone seems to offer less opportunity for variation to the particular organisms under observation than do the other peptones which were employed, when used in the method prescribed by Clark and Lubs.

3. The advantage of Witte's peptone over the others is, however, comparatively small, in so far as these experiments show.

4. The final hydrogen-ion concentration reached by the several organisms in the different peptones is variable to a certain extent, and as far as can be seen from the data on hand, the method of cultivation, whether it be in tubes or in bottles, seems to influence the acid-production differently in different peptones, although the differences are slight.

5. A study of the nitrogen metabolism of the organisms, with a determination of the amino-acids necessary for their welfare, and a study of the influence of these amino-acids on the type of fermentation which predominates would be of great help in the investigation of variability of the cloacae-aerogenes group of bacteria.

6. Meat extract seems to contain some substances which are desirable as food for the organisms, because of the frequent increase of sugar utilization in the media to which it has been added.

7. A very strong Voges and Proskauer reaction is given in a phosphate-meat-extract-glucose medium which is apparently more reliable than the methyl red test, and on account of its lesser variability the Voges and Proskauer reaction is preferable.

8. The high ratio group organisms grow well in Uschinsky's medium containing glucose instead of glycerin, and give a Voges and Proskauer reaction. The low ratio organisms grow in it with difficulty.

QUALITATIVE CHARACTERIZATION OF ORGANISMS IN THE PRESENT COLLECTION

Since the biometric method has failed as an important aid in the classification of the organisms, recourse must be had to the older methods of identification based on qualitative differences. All that has been gained from the biometric studies here recorded may be summed up as follows:

There seem to be 2 subgroups of organisms which comprise the colon-aerogenes group. One of these is always methyl red positive and Voges and Proskauer negative, these characters being stable. The other group is variable, but its members usually are methyl red negative and Voges and Proskauer positive. Table 6 presents a system of classification with these 2 major groups as the basis.

The group number offers a convenient and easy method of recording qualitative differences, and of studying the numbers of organisms presenting similar characters. All organisms showing gas, although subsequent titration might show the culture to be alkaline, were considered as acid- and gas-formers, on account of the double reaction in

the sugar decomposition of the high ratio group. This idea was suggested by Rogers, Clark, and Evans.[8]

It is necessary to recall again that in this collection of cultures there are a few that come from sources which are questionable, that is, in which there may have been a possibility of pollution. These are designated as 'Discards.' Those which would be acceptable from the sanitary standpoint are designated as 'O. K.'

TABLE 6

DISTRIBUTION OF THE ORGANISMS OF THE PRESENT COLLECTION IN 13 OF THE GROUP NUMBERS OF THE CARD OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

Group Number	Total Number Cultures	Number O. K.	Number Discards	Methyl Red Positives O. K.	Methyl Red Positives Discards	Probable Name of Group
(1) 121.1112011	9	9	0	0	..	?
(2) 221.1113031	22	12	10	6	8	?
(3) 221.1113032	124	93	31	0	0	B. cloacae Rogers Group D
(4) 221.1113532	13	10	3	0	0	Rogers Group C
(5) 221.1133032	2	1	1	0	0	?
(6) 221.1213031	2	0	2	..	2	?
(7) 221.1313032	1	1	0	0	0	Proteus ?
(8) 222.1112031	1	1	0	1	..	?
(9) 222.1113031	11	9	2	4	0	B. coli communior Rogers Group D
(10) 222.1113032	2	2	0	0	0	Rogers Group D
(11) 222.1133031	13	13	0	13	0	B. coli communis and acidi lactici
(12) 222.1133033	1	1	0	1	..	?
(13) 222.1212031	1	1	0	1	..	?
Totals.....	202	153	49	26	10	

It can be seen from Table 6 that there is a very marked correlation between certain groups and the methyl red reaction, especially in the cloacae and in the colon-acidi lactici group.

Rogers, Clark, and Evans,[8] say, ". . . Species so established cannot be identified or separated from one another by the turn of a single characteristic. Natural groups of bacteria are bound together by the common possession of a certain combination of characteristics, no one of which is absolutely fixed."

According to this assumption, many of the group numbers which are frequently represented by very few organisms which differ from other group numbers in only 1 or 2 characters, but have in common many more characters, might be combined in several larger groups. (Of course, the observations of Rogers and his associates would necessitate keeping the organisms of presumably the same gas ratio together.)

On this basis the foregoing groups, numbered 1-13, consecutively, were regrouped into the following 6 'Types,'* as shown in Table 7.

TABLE 7

DISTRIBUTION OF THE 13 ORGANISMS OF THE PRESENT COLLECTION INTO 6 TYPES

All Sporeformers: Type I		121.1112011	No. of Representatives 9
High Ratio Organisms: Type	Group		
II	2	221.1113031	8
	3	221.1113032	124
	4	221.1113532	13
	5	221.1133032	2
	7*	221.1313032	1
III	9	222.1113031	7
	10	222.1113032	2
Low Ratio Organisms: Type	Group		
IV	2	221.1113031	14
	6	221.1213031	2
V	8	222.1112031	1
	9	222.1113031	4
	13	222.1212031	1
VI	11	222.1133031	13
	12	222.1133033	1

* This has the group number of the proteus group but gives the Voges and Proskauer reaction, and is therefore included here.

To make the data in Table 7 more intelligible to those who are not accustomed to using the group number, the graphic and tabular method adopted by Rogers and his associates has been utilized. Because all of the cultures fermented glucose this sugar was not included among the tests.

Certain correlations may be noted: Of the 36 low ratio organisms, 26 produce indol, and 35 ferment glycerol with gas-production. In the whole collection exclusive of the known stock cultures there are 59 strains which can ferment glycerol with gas-production; they are distributed through the several types as shown in Table 9.

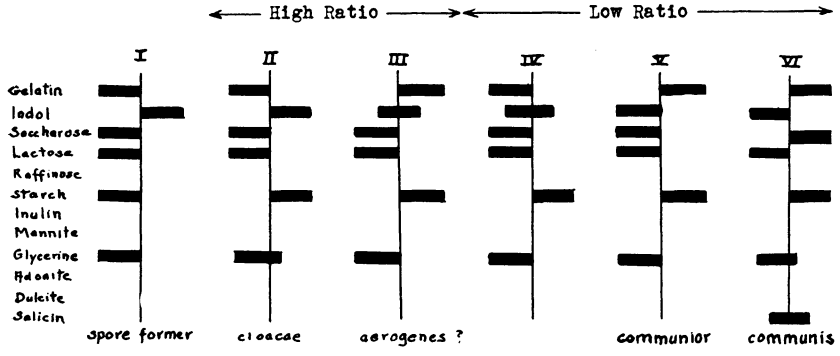
SUMMARY OF QUALITATIVE STUDIES

Thirteen group numbers have been represented by the collection of gas-formers found in nature. These have been regrouped into 6 types on the basis of the work of Rogers and his associates, in which acid-production from a sugar has been classed with acid and gas-production under the term 'fermentation.'

* In working out these groups the known stock cultures were omitted.

TABLE 8

GRAPHIC AND TABULAR METHOD OF DISTRIBUTION OF ORGANISMS



	Liquefaction		Decomposition of				
Type	Gelatin	Indol	Saccharose	Lactose	Starch	Glycerol	Salicin
I	9 100%	0 0%	9 100%	9 100%	9 100%	9 100%	
II	148 100%	2 1.3%	146 98.5%	147 99%	0 0%	148 100%	
III	0 0%	4 45%	9 100%	9 100%	0 0%	9 100%	
IV	16 100%	6 37.5%	16 100%	16 100%	0 0%	16 100%	
V	0 0%	6 100%	6 100%	6 100%	0 0%	6 100%	
VI	0 0%	14 100%	0 0%	14 100%	0 0%	13 93%	7 50%

TABLE 9

DISTRIBUTION, ACCORDING TO TYPE OF STRAINS WHICH CAN FERMENT GLYCEROL WITH GAS-PRODUCTION

Type	Number of Strains	Cultures Positive, %
I	9	100
II	8	5.4
III	7	77.9
IV	16	100
V	6	100
VI	13	93
Total	59	

All of these 6 types may not be valid natural groups, especially those having few representatives, but there is reason to believe in the validity of Type I, the spore formers; Type II, the organisms of the cloacae-aerogenes group; and Types V and VI, which are the colon group proper.

Type II contains both pigment and nonpigment formers which give approximately the same sugar fermentations. The single representative of the group number 221.1313032 was included with this type, even though it does not utilize lactose, because it did not seem advisable to create another type for a single organism. (At this point it might be remarked that no recognizable members of the large proteus group have been encountered. Their habitat seems to be elsewhere than in the soil. From our experience and the publications of Kendall and his associates we infer that B. proteus (vulgaris) would be found with the low ratio organisms and would differ from them only in regard to the lactose fermentation.) The single organism mentioned above is a high ratio organism, gives a positive Voges and Proskauer reaction, and hence is in all probability not a member of the proteus group. Type II and Rogers, Clark, and Evans Group C are probably the same.

Type III should contain members of the B. [lactis] aerogenes group (nonliquefiers producing gas from glycerol). Seven representatives of Number 222.1113031 have been met with which are high ratio. These are probably the same as Rogers, Clark, and Evans Group 'D.' It is noteworthy that so few of the aerogenes group have been found in this research as compared with the large number of the cloacae group. In their paper, Rogers, Clark, and Evans report 40 cultures belonging to Group C and 90 to Group D. In our work we have found 148 and 9, respectively, in the groups which we believe correspond to their C and D groups. This suggests a different habitat for B. cloacae than for B. aerogenes. It may be, however, that an error in the determination of gelatin liquefaction accounts for the differences in the proportion of cultures in the 2 groups. There is no doubt that all of the liquefiers in this research are liquefiers, whereas incubation for 20 days at 20 C. leaves some doubt about the status of those which have not liquefied.

Type IV may be one of the types which would be classed with the colon bacillus by the ordinary methods of identification. At present nothing can be said of it to correlate it with some known group of organisms except that it has all of the reactions of the colon group, but yet liquefies gelatin.

Type V is probably the well known B. [coli] communior group.

Type VI probably contains members of the B. [coli] communis and B. acidi lactici groups, although the cretaceous growth of certain of them leaves doubt as to their identity.

It is quite evident that B. cloacae is the predominating soil gas-former, in so far at least as the data here are concerned.

GENERAL SUMMARY

About 1000 samples of soils, twigs, leaves, flowers, etc., have been examined for the presence of gas-formers. The organisms collected were arranged in 2 groups, one of which came from places where there is no reason to suspect fecal pollution of any sort, and the other from sources in which pollution is not an improbability. The organisms have been classified together and also separately.

An effort was made to study the cultures by the biometric method. Methods of cultivation and titration were given a critical study and attempts made to standardize them. Variability was encountered in the high ratio organisms to such an extent that the biometric method was of very little value in the classification work. This led to a study of the variability and metabolism of the high ratio organisms. A theory of the mechanism of the variability was formulated based on the apparent independence of 2 simultaneous methods of sugar decomposition. The correlations of the methyl red and the Voges and Proskauer reactions are readily explained by this theory, and the experimental results of the research appear to confirm the observation of Levine on this correlation.

Efforts to bring about mutation of the high ratio groups were unsuccessful.

The Voges and Proskauer reaction is apparently more dependable than the methyl red test.

The influence of specific peptones on the variability and the methyl red test has been studied, and while no very definite conclusion has been reached it was found that the peptone showing the lowest variability gave a medium of lowest amino-acid content.

It was shown that the substitution of meat extract alone for peptone in Clark and Lubs' medium gave somewhat variable methyl red tests, but the Voges and Proskauer reaction under such conditions was so strong as to be a deep red, instead of the customary pink obtained with peptones.

Uschinsky's medium minus glycerol, but with glucose added, furnished proper conditions for a Voges and Proskauer reaction, but is unsuited for the methyl red test. Low ratio organisms give very poor growth in this medium, while those in the high ratio group find it favorable for rapid multiplication.